Early-life gut microbiota and neurodevelopment in preterm infants: any role for Bifidobacterium?

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Abstract

Despite the well-recognized importance of proper gut microbiota assembly for the child's future health, the connections between the early-life gut microbiota and neurocognitive development in humans have not been thoroughly explored so far. In this pilot observational study, we aimed to unveil the relation between dynamic succession of the gut microbiota in very low birth weight infants during the first month of life and their neurodevelopment, assessed at 24-month corrected age. According to our data, the early-life gut microbiota of preterm infants with normal vs impaired neurodevelopment followed distinct temporal trajectories with peculiar compositional rearrangements. In this context, early *Bifidobacterium* deficiency seem to constitute a negative biomarker of adverse neurological outcomes.

Conclusion: our data might pave the way for future in-depth studies focusing on potential impact of bifidobacteria or specific microbiota patterns on neonatal neurodevelopment and lay the foundation for microbiome-based clinical practices to modulate altered profiles and improve long-term health.

Keywords: very low birth weight; preterm infants; gut microbiome; neurodevelopment; *Bifidobacterium*.

What is Known:

- Preterm infants are at increased risk for adverse neurological outcomes and gut microbiota dysbiosis.
- The gut microbiota and the nervous system share critical developmental windows in early life.

What is New:

- The absence of *Bifidobacterium* at 30 days of life in preterm infants is associated with neurodevelopment impairment in early childhood.
- The administration of *Bifidobacterium* strains could promote optimal neurocognitive development in fragile infants.

Abbreviations

CA: correct age

GQ: general development quotient

IQR: interquartile range

NI: neurodevelopmental impairment

VLBW: very low birth weight

Introduction

Preterm infants are at increased risk for adverse neurological outcomes and gut microbiota dysbiosis [1]. While the association of gut microbiota dysbiosis with short-term clinical outcomes is widely studied, its relationship with long-term outcomes remains largely unknown. Interestingly, the gut microbiota and the nervous system share critical developmental windows in early life. Recently, the French EPIFLORE prospective observational cohort study on very preterm newborns found out that the gut microbiota at week 4 after birth exhibited bacterial patterns that varied according to gestational age, perinatal characteristics, individual treatments, and neonatal intensive care unit strategies; furthermore, early gut microbiota features were associated with 2-year outcomes, even after adjustment for confounders [2]. While animal model studies have shown a direct connection between early-life microbiota and neurocognitive development, data in humans are scarce. Therefore, our aim was to investigate associations between gut microbiota dynamic features during the first month of life in very low birth weight (VLBW) preterm infants and neurodevelopment in early childhood.

Materials and methods

Preterm infants with gestational age <32 weeks and/or VLBW were enrolled after birth and followed longitudinally up to 24-month corrected age (CA) within a prospective pilot observational study. Stool samples were collected at 1, 4, 7, and 30 days of life. Microbial DNA was subjected to 16S rRNA Illumina sequencing as previously described [3]. Bioinformatics and statistics are detailed in Supplementary Methods. Neurodevelopment was assessed at 24-month CA by revised Griffiths Mental Development Scale (GMDS-R), as a part of neurodevelopmental follow-up of preterm infants. The psychologist performing the Griffiths Mental Development examination were blinded to microbiota analysis. GMDS-R General Development Quotient (GQ) was calculated using standardized score tables for the English infant population (mean ± SD, 100.5 ± 11.8), as no standardized data are available for the Italian population. Normal development was defined as a GQ score ≥88.7, and cut-offs for mild or moderate/severe neurodevelopmental impairment (NI) were 88.6 and 76.8, respectively [4]. The Ethical Board of S. Orsola Hospital (Bologna, Italy) approved the study (study ID 25/2014/U/Oss) and written informed consent was obtained from infants' parents.

Results

Twenty-seven preterm infants were recruited (14 female [51.9%], 21 born to Caucasian mothers [77.8%], 2 to Asian mothers [7.4%], 4 to African mothers [14.8%]). Median (interquartile range - IQR) gestational age was

30.6 (28.6-33.6) weeks and median (IQR) birth weight 1,196 (917-1,374) g. At 24-month CA, 21 infants had normal neurodevelopment and 6 showed NI (3 mild and 3 moderate/severe NI cases). Infants with NI had higher need for surfactant administration. No other difference in clinical characteristics was described between infants with vs. without NI. Detailed clinical characteristics of the recruited infants stratified by neurodevelopmental outcome at 24 months are shown in **Table 1**.

As for microbiota assessment, no significant differences were found in GM alpha diversity between study groups over time, except for a trend towards greater diversity in infants with NI at day 30 (p=0.17, Wilcoxon test) (**Figure 1a**). On the other hand, beta diversity analysis revealed distinct temporal trajectories between infants with NI and those with normal neurodevelopment ($p \leq 0.05$, PERMANOVA) (**Figure 1b**). Furthermore, based on the unweighted UniFrac metrics, at day 1 and 30, there was significant segregation between the two types of NI (mild vs moderate/severe, $p \leq 0.046$). At the taxonomic level (**Figure 1c**), compared to infants with normal neurodevelopment, those with NI tended to be enriched in *Enterococcaceae* at day 7 and 30 (p=0.2, Wilcoxon test). Interestingly, despite an early overrepresentation of *Bifidobacteriaceae* in the gut microbiota of infants with NI (p=0.05), their levels cleared by day 7 and tended to be lower than those of infants with normal neurodevelopment at day 30 (p=0.1). Notably, at day 30, *Bifidobacterium* abundance was positively correlated with the 24-month GQ score (p=0.01, tau=0.449; Kendall rank correlation test) (**Figure 1d**). The major represented species were *B. longum* and *B. breve*, neither of which were found in the gut microbiota of infants with NI (**Figure 1e**).

Discussion

Through this prospective pilot observational study, we shed some light on the connections between the early-life gut microbiota dynamic assembly and neurocognitive development of preterm infants in early childhood. In particular, we found a relationship between both dynamic patterns (i.e., beta diversity trajectories) and static features (i.e., relative taxon abundance at certain timepoints) of the gut microbiota during the first month of life with neurodevelopmental outcomes at 24-month CA. Our findings appear to be in line with those of the recent EPIFLORE study, showing that early microbiota is associated to later neurodevelopment [2]. Very recently, a systems-level analysis of the gut microbiota, immune system, and neurophysiological development during hospitalization up to term equivalent age of 60 extremely preterm infants (with gestational age <28 weeks and birth weight < 1000g)

revealed that *Klebsiella*-dominated gut microbiota communities are highly predictive for brain damage and are associated with a pro-inflammatory immunological profile [5]. This study suggested that aberrant development of the gut-microbiota-immune-brain axis could contribute to the onset and/or aggravation of brain injury in extremely preterm infants. To the best of our knowledge, our study is the first study reporting on the association between early colonization with *Bifidobacterium* in preterm infants and neurodevelopment in early childhood: specifically, the absence of *Bifidobacterium* at 30 days of life appeared to be associated with NI. *Bifidobacterium* spp. are known to play a pioneering role in the healthy development of the infant gut microbiota, contributing to the fine-tuning of the immune system and potentially exerting neuroprotective effects, mainly through the modulation of the production and release of neuroactive substances [6, 7]. The absence and/or low abundance of *Bifidobacterium* might thus constitute a biomarker of vulnerability and immaturity, and this observation could potentially lead to early intervention strategies aimed at promoting optimal neurodevelopment in preterm infants during neonatal intensive care unit hospitalization and after discharge. Some limitations of our study need to be acknowledged, especially the small number of subjects included in our monocentric cohort. Furthermore, another limitation is constituted by the time window of microbiota analysis, as stool samples were collected only at days 1,4,7, and 30, making us blind to microbiota changes after the first month of life.However, although preliminary, we believe that the results of the present study are promising. Further studies in larger cohorts, possibly with other omics techniques (e.g., metagenomics and metabolomics) and animal models, are needed to provide additional evidence and mechanistic insights. Once the role of *Bifidobacterium* in promoting optimal neurocognitive development in preterm infants is confirmed, it would be reasonable to design further trials evaluating microbiome-based clinical practices, including both microbiome-modifying strategies and the use of *Bifidobacterium* strains as probiotics, aimed at modulating unbalanced profiles and favoring the long-term health of these fragile infants.

Declarations

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Conflicts of interest/Competing interests: The authors have no conflicts of interest to declare that are relevant to the content of this article.

Availability of data and material: Raw sequencing reads are available in the National Center for iotechnology Information Sequence Read Archive (Bioproject ID PRJNA783925) .

Code availability: Not applicable.

Authors' contributions: AA, PB and LC designed the study protocol. IB, MB, ST, EB, and AS performed data acquisition and analysis. IB, MB, ST, and AA wrote the first draft of the manuscript, which was revised critically by AS, EB, PB, and LC. All the authors gave final approval of the version to be submitted and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval: The Ethical Board of S. Orsola Hospital (Bologna, Italy) approved the study (study ID 25/2014/U/Oss). The study was performed in accordance with the ethical standards of the Declaration of Helsinki.

Consent to participate: Written informed consent was obtained from the parents.

Consent for publication: Parents signed informed consent regarding publishing their data.

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Figure legends

Figure 1 Early-life gut microbiota assembly in very low birth weight infants with normal or impaired neurodevelopment. a, Boxplots showing alpha diversity, measured according to the inverse Simpson index, in stool samples form preterm infants with neurodevelopmental impairment (NI_yes) or with normal neurodevelopment (NI_no), collected on days 1, 4, 7 and 30 of life. #, p=0.17; Wilcoxon test. **b**, Principal Coordinates Analysis (PCoA) based on weighted (left) and unweighted (right) UniFrac distances, showing all samples colored by time point. Symbols indicate the presence or absence of NI, and the arrows represent the direction of temporal variations of the gut microbiota in each study group. **c**, Boxplots showing the relative abundance distribution of bacterial families differentially represented between study groups over time. *, p=0.05; #, $p \leq 0.2$; Wilcoxon test. **d**, Scatter plot of correlation between *Bifidobacterium* relative abundance at day 30 and General Development Quotient score at 2 years of corrected age (p=0.01, tau=0.449; Kendall rank correlation test). **e**, Hierarchical Ward-linkage clustering based on Kendall correlation coefficients of the relative abundance of *Bifidobacterium* spp. in stool samples from preterm infants at 30 days of life. Samples are color-coded by study group in the vertical bar (same colors as panel A). *, unclassified species

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202 **Table 1. Characteristics of the study population stratified by neurodevelopmental outcome at 24-month**

203 **corrected age.**

Variable	Normal neurodevelopment (n=21)	Neurodevelopmental impairment (n=6)	p-value
Mother's origin Italy, *No.* (%)	15 (71)	3 (50)	0.37
Female, *No. (%)*	12 (57)	2 (33)	0.38
Birth weight, *median (IQR), g*	1200 (1041-1385)	909 (800-1389)	0.21
Gestational age, *median (IQR), weeks*	30.6 (28.6-33.7)	29 (26.2-32.3)	0.43
Culture proven sepsis, *No. (%)*	0	1 (17)	0.22
Necrotizing enterocolitis, *No. (%)*	0	0	
Respiratory distress syndrome, No. *(%)*	15 (71)	6 (100)	0.28
Surfactant administration, *No. (%)*	4 (19)	4 (67)	*0.04*
Intraventricular haemorrhage, *No. (%)*	1 (5)	1 (17)	0.40
Patent ductus arteriosus, *No. (%)*	4 (19)	2 (33)	0.59
Exclusive human milk during first week, *No. (%)*	19 (90)	5 (83)	0.55
Exclusive human milk during first month, *No. (%)*	18 (86)	3 (50)	0.10

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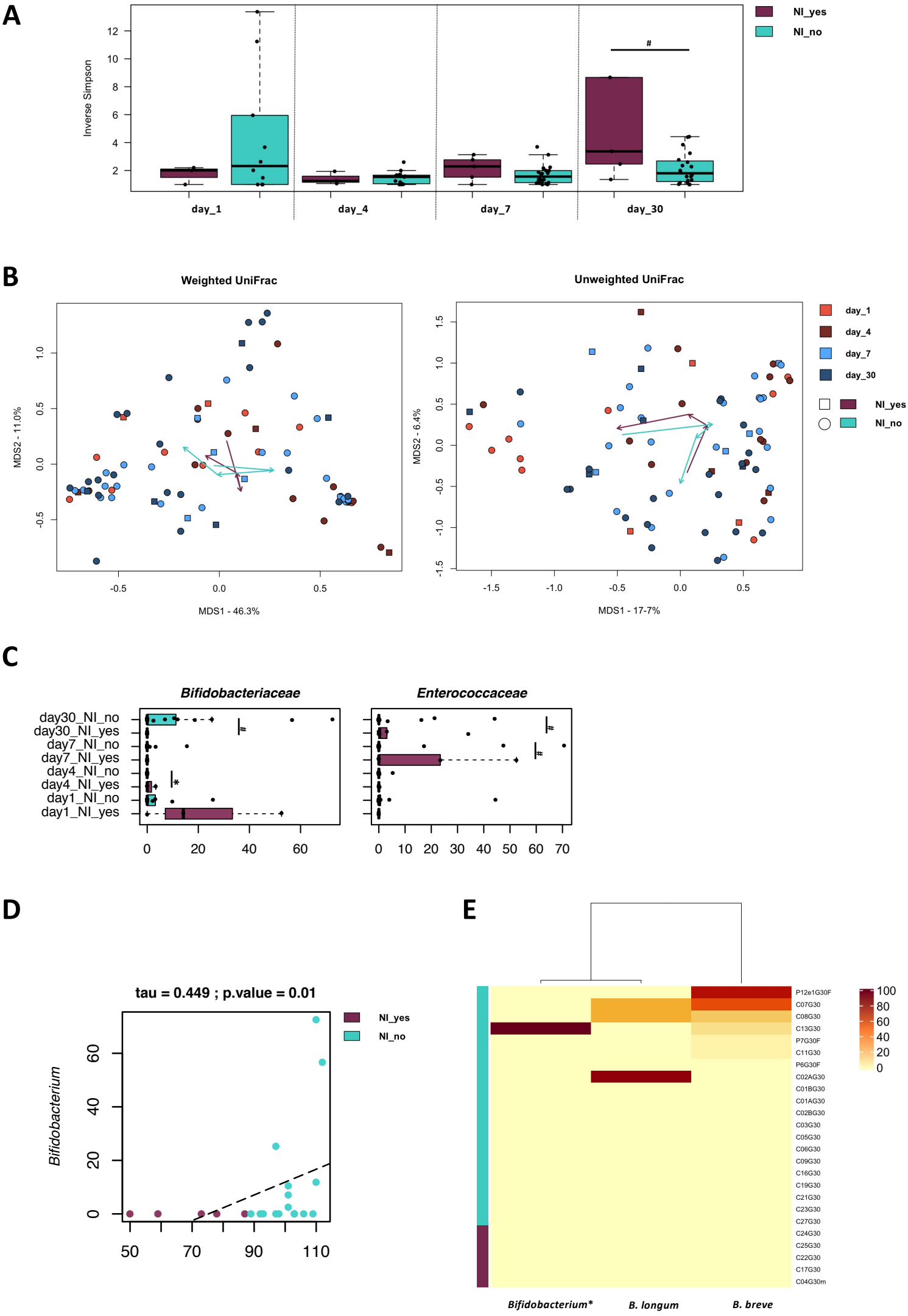
A
Inverse Simpson
NI_yes
NI_no
#
day_1
day_4
day_7
day_30
B
Weighted UniFrac
Unweighted UniFrac
MDS2 - 11.0%
MDS1 - 46.3%
MDS2 - 6.4%
MDS1 - 17-7%
day_1
day_4
day_7
day_30
NI_yes
NI_no
C
Bifidobacteriaceae
Enterococcaceae
day30_NI_no
day30_NI_yes
day7_NI_no
day7_NI_yes
day4_NI_no
day4_NI_yes
day1_NI_no
day1_NI_yes
D
tau = 0.449 ; p.value = 0.01
Bifidobacterium
NI_yes
NI_no
E
Bifidobacterium*
B. longum
B. breve